**Regulation of hyaluronan biosynthesis and clinical impact of**

**excessive hyaluronan production**

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**Abstract**

The tightly regulated biosynthesis and catabolism of the glycosaminoglycan hyaluronan, as well as its role in organizing tissues and cell signaling, is crucial for the homeostasis of tissues. Overexpression of hyaluronan plays pivotal roles in inflammation and cancer, and markedly high serum and tissue levels of hyaluronan are noted under such pathological conditions. This review focuses on the complexity of the regulation at transcriptional and posttranslational level of hyaluronan synthetic enzymes, and the outcome of their aberrant expression and accumulation of hyaluronan in clinical conditions, such as systemic B-cell cancers, aggressive breast carcinomas, metabolic diseases and virus infection.

**Introduction**

Tightly regulated communication between cells and extracellular matrix (ECM) components controls embryonic development, as well as tissue homeostasis in the adults. During tumorigenesis and cancer progression, as well as inflammation, an excessive remodeling of the ECM composition occurs, promoting tumor growth, invasion and metastasis [1, 2]. Under such conditions, aberrant signaling promotes epigenetic gene alterations and the expression of growth factors and cytokines that trigger the expression of key regulatory proteins and ECM molecules.

Hyaluronan, a ubiquitous glycosaminoglycan, is an important constituent of the ECM which plays a central role during embryogenesis [3, 4], inflammation and cancer [5-7]. It has been ascribed both a structural role through its lubricating and water-retaining properties creating flexibility in matrices, and a cell signaling role modulating cell proliferation, invasion and metastasis through its interactions with hyaluronan binding proteins [4, 8-11].

The metabolism of hyaluronan is carefully regulated by synthetic (HAS1, HAS2, HAS3) and catabolic (HYAL1, 2, PH-20, KIAA1199 and TMEM2) [6, 12, 13] enzymes. Moreover, hyaluronan is taken up by cell surface receptors, such as CD44, if expressed at its functional “state” with respect to uptake, internalization and degradation. For example, hormone negative aggressive breast cancer cells constitutively express CD44 that recognize and bind exogenous added hyaluronan, but only minute amounts of newly synthesized hyaluronan is taken up by CD44, internalized and degraded [14]. In contrast, in alveolar macrophages CD44 functions as a scavenger receptor, which internalize hyaluronan and deliver it to lysosomes for degradation [15, 16]. In addition to HYALs activities, the size of hyaluronan is modified by reactive oxygen species (ROS), which are constantly produced in the body and can cause fragmentation of hyaluronan [17]. About 30% of about 15 g hyaluronan that is present in an adult human is degraded locally through the orchestrated actions of hyaluronidases and ROS, whereas the remaining 70% is carried by the lymph to the lymph nodes where the major part is degraded by the lymphatic vessel endothelial cells, after binding and internalization by the hyaluronan receptor LYVE-1; only a small part is carried to the bloodstream, most of which is degraded by liver endothelial cells [17-20].

Accumulating evidence link increased hyaluronan deposition to severity of diseases, such as rheumatoid arthritis, lung diseases and cancer. In order to elucidate the biological functions of hyaluronan, it is important to understand its biosynthesis, uptake and turnover, and signaling. In this review, we will focus on the mechanisms underlying hyaluronan biosynthesis in mammalian cells. A better understanding of hyaluronan biology may facilitate the design of therapeutic strategies and improve the outcome of patients.

1. **Distribution, interactions and functions of hyaluronan**

Hyaluronan is comprised of repeating disaccharide units of glucuronic acid (GlcUA) and N-acetyl glucosamine (GlcNAc) [21, 22], and is present in essentially all tissues (in particular skin, around blood vessels and lung bronchioles) and body fluids. Under physiological conditions, hyaluronan of high molecular mass, of about 1x106 Da, is one of the prominent components of intercellular as well as pericellular (glycocalyx) matrices. Newly synthesized hyaluronan, in pericellular matrices surrounding mesenchymal and mesothelial cells, is attached to the membrane-bound hyaluronan synthesizing enzymes (HAS1, 2 and 3), whereas it is preferentially attached to its cell-surface receptors including CD44 and/or RHAMM (known also as CD168) around chondrocytes, hematopoietic, epithelial and endothelial cells [23-26] . The interactions between hyaluronan and hyaluronan binding proteins, such as versican or aggrecan, create the stability and flexibility of pericellular as well as intercellular matrices, and also transmit signals through hyaluronan receptors, e.g. CD44 [27], RHAMM [28] and LYVE-1 [5, 29-31]. Hyaluronan is not only found in pericellular and extracellular matrices, but is also found intracellularly where it affects mitosis and migration because of its association with the mitotic spindle, RHAMM and microtubules [32, 33].

During inflammatory processes, hyaluronan is captured by CD44 and/or RHAMM to assemble with the inflammation-induced TSG-6 molecules that bind hyaluronan and the heavy chain of inter--trypsin inhibitor (II), resulting in the deposit of hyaluronan cables. Such cable-like structures comprised of hyaluronan and versican can serve as a platform for inflammatory cells, as well as to provide cues to initiate repair responses and regulate inflammation [9, 30, 34]. In particular, studies by de la Motte and colleagues have highlighted a regulatory role of hyaluronan in normal gastrointestinal tract physiology and after challenges by pathogens. For example, in ulcerative colitis, alterations in hyaluronan distribution and levels of deposition was observed as well as increased leukocyte recruitment resulting in persistent inflammation [35, 36]. Notably, low molecular mass hyaluronan (about 35 000 Da) was shown to contribute to intestinal epithelium innate defense by promoting the expression of -defensin 2 in a TLR4-dependent manner [37]. Importantly, this small-sized hyaluronan also functions as an anti-inflammatory mediator by suppressing hepatic and intestinal injury; small-sized hyaluronan normalized over-activated Kupffer cells by restoring their miR-181b-3p expression which modulates the expression of importin 5 and sensitivity to TLR4 signaling [38]. Most likely, deposition of HAS2- and HAS3-synthesized hyaluronan by gut tissue and the neuromuscular compartment plays a regulatory role in inflamed intestine [35, 39]. Thus, dependent on its size and interactions with hyaluronan binding proteins and receptors, hyaluronan can both restore and resolve inflammation, as well as foster inflammation followed by interstitial edemas and organ dysfunction [8, 16, 35, 40-44].

Eva Turley and co-authors have launched the term “portable cancerized microenvironment” referring to pericellular hyaluronan-rich matrices protecting circulating tumor cells from apoptosis and promoting their metastasis [45]. Furthermore, hyaluronan-containing pericellular matrices can protect cells from viral infections, and promote the dissemination of breast cancer cells by modulating cellular adhesion/de-adhesion and modulate glycocalyx permeability [25, 46-48].

The roles of hyaluronan in disease are regulated at many levels that we know very little about. A huge diversity of signaling networks have been identified, through which hyaluronan via its receptors affects pathologic events including breast cancer progression and the severity of inflammation and virus infection. The capability of each HAS isoform to synthesize hyaluronan is cell specific and dynamically regulated at transcriptional, post-transcriptional and post-translational levels, in a context-dependent manner. Thirty years after the cloning of the HAS isoforms, their relevance for excessive hyaluronan production in pathological disorders is still not completely understood.

2. **Mechanisms of regulation of hyaluronan biosynthesis**

**2.1 Regulatory elements that control transcription of HAS isoforms**

In adults, elevated hyaluronan levels are associated with disease progression. Injured, inflamed or cancer stroma tissues are rich sources of cytokines and growth factors that differentially induce the transcription of the HAS isoforms [6, 49-56]. The human HAS1, HAS2 and HAS3 isozymes (in mouse designated Has1, Has2 and Has3, respectively) [22] are located on chromosomes 19q13.41, 8q24.12, and 16q22.1, respectively. Gene duplication events and divergent evolution resulted in the hyaluronan synthase gene family; ancestral HAS though a duplication event gave rise to HAS1 and ancestral HAS2, which after another duplication event gave rise to HAS2 and HAS3. The HAS isoforms show different expression patterns and functions [4, 57-61]. *HAS1* and *HAS3* are comprised of five transcripts each, whereas *HAS2* gene has one transcript and a *HAS2* antisense RNA (*HAS2-AS1*) [62] (www.ensembl.org). *HAS2-AS1* is a long non-coding RNA (lncRNA) which is transcribed on the opposite strand to *HAS2* and exhibits complementarity with the *HAS2* untranslated exon 1. It is becoming clear that lncRNAs, through their ability to regulate target DNA, RNA and protein at the pre- and posttranscriptional level, play important roles in the control of cellular fate [63]. *HAS2-AS1* inhibits *HAS2* mRNA expression in osteosarcoma cells [62], but stabilizes and increases *HAS2* mRNA expression in human kidney proximal tubular epithelial cells and human aortic smooth muscle cells [64, 65]. The reason for the different effects of *HAS2-AS1* remains to be elucidated. Notably, the promoter region of *HAS2-AS1* possesses a binding site for O-GlcNAcylated nuclear factor B/p65 (NF-B) [65] and hypoxia-responsive element (HRE) [66] (see Fig. 1 for additional information on response elements and references). Recently, the IL1/PDGF-responsive smooth muscle enriched lncRNA (*SMILR*) located on chromosome 8 only 750 kbp from the *HAS2* gene, was shown to be important for HAS2 expression and vascular smooth muscle cell proliferation. Notably, *SMILR* expression has been linked to inflammatory vascular pathologies [67].

Several observations support the notion that the complexity of the transcriptional control of HAS isoforms varies in different cell types. Studies on the promoter sequences of each *HAS* gene, using *in silico* and experimental data, identified transcription factor binding sites, including cAMP-responsive element binding proteins (CREB); NF-B binding sites; GC-rich motifs for binding of SP1 transcription factor; and E2F-*myc* binding sites that might link HAS1 and/or HAS2 expression to the cell cycle [68]. Further studies on the promoter region of *HAS2* resulted in the identification of evolutionary conserved binding sites for the transcription factors SP1, NF-Y/CCAAT and NF-B, in human, mice and equine genomic DNAs [69].

Epidermal growth factor (EGF) and *all-trans*-retinoic acid (RA) powerfully influence wound healing and various skin diseases, involving HAS2-mediated hyaluronan production [70]. Binding elements for STAT3 which translocates to the nucleus and binds to its response elements after EGF stimulation, and RA, were identified in the promoterof *HAS2*. Notably, the STAT3 element overlaps with a putative binding site for NF-B which is also activated by the EGF/Ras signaling pathway, and by TNF- [71, 72]. Furthermore, the *HAS2* gene, which displays a rapid response to forskolin, causing increased level of cAMP and thus activating CREB1, is regulated by convergence of CREB and RA signaling that might fine-tune *HAS2* induction [73]. Further studies revealed that SP1 and Sp3 mediate the constitutive transcription of the *HAS2* gene [74] (Fig. 1). Interestingly, Sp factors and STAT3, as well as RA elements, affect each other´s activities, thus further providing insights into the complex regulation of the *HAS2* gene. In addition, the Yin-Yang 1 (YY1) transcription factor, which increases in response to glucose binds to response elements on the promoter of *HAS2* gene, suggesting a link to cell metabolism [75].

Studies on the cytokine-mediated transcriptional induction of *HAS1* revealed that TGF1 induces *HAS1* via Smad3, but not Smad2, while IL-1-mediated *HAS1* up-regulation was mediated via Sp3, but not SP1, in dermal fibroblasts [76]. Given that TGF1 is a key promoter of fibrosis and IL-1a key inflammatory mediator, this up-regulation of *HAS1* may contribute to fibrotic/inflammatory diseases.

Recent analysis of the promoter region of *HAS3* revealed that the *HAS3* promoter is TATA-box less and has two transcription start sites. It contains a GC box (SP1 binding site is important for *HAS3* activity), and other binding sites for the transcription factors NF-B and CCAAT/Enhancer binding protein (C/EBP), which is known to regulate genes involved in immune and inflammatory responses [77, 78] (Fig. 1). Most recently, the Np63 transcription factor, which belongs to the p53 family, occupy a p63-binding site (p63 BS) in the *HAS3* promoter promoting its expression, and contributing to proproliferative and prosurvival pathways in head and neck squamous cell carcinoma [79].

The identification and characterization of human *HAS1, 2 and 3* promoters provide a basis for understanding their regulation during pathophysiological conditions.

**2.2 Regulation of hyaluronan synthesis at the transcriptional level by growth factors, cytokines, prostaglandins and hormones**

During tissue injury, inflammation and cancer, accumulation of hyaluronan of a polydisperse size has been seen (average molecular mass of 500 kDa), resulting in the remodeling of the ECM and promoting inflammation and transformation. Hyaluronan is extruded through pores, created by plasma membrane-associated homo- or hetero-oligomers of HAS isoforms, in conjuction with its synthesis [80-82]. The hyaluronan synthesizing enzymes have similar structures and amino acid sequences; the sequence identities are 55% for HAS1/HAS2, 57% for HAS1/HAS3 and 71% for HAS2/HAS3, and homologous isoforms between human and mice share about 99% sequence identity [22]. The expression patterns of the HAS isoforms differ in adult tissues, suggesting the existence of a variety of local secreted regulatory stimulants that affect differentially *HAS* mRNA and protein stability [49, 83]. Thus, a better knowledge of the intracellular signaling pathways, leading to up- or down-regulation of HAS isoforms, is important to identify methods to inhibit hyaluronan synthesis in diseases where hyaluronan is overproduced.

Studies by us and others have demonstrated that growth factor- and cytokine-mediated induction of HAS isoforms, and subsequently hyaluronan production differs dependent on the cell type. For example, PDGF-BB, which is a potent mitogenic and chemotactic agent, exerted its hyaluronan stimulatory effect primarily through the induction of *HAS1*and *HAS2* mRNA in mesothelial cells, but through the induction of all three synthases in dermal fibroblast cultures, via activation of the ERK1/2 MAP-kinase and PI3K/Akt signaling pathways (Table 1) [49, 51]. Interestingly, treatment of mesothelial cells with physiological concentrations of hydrocortisone, down-regulated the transcriptional activity of *HAS2*, whereas it slightly suppressed the *HAS1* transcript and had no effect on *HAS3* mRNA [49]. Similarly, PDGF-BB-mediated *HAS2* induction and increased hyaluronan was observed during the detachment phase of proliferative and migrating arterial smooth muscle cells [84]. Wound healing, which is commonly associated with increased hyaluronan synthesis, is characterized by overlapping phases of inflammation, proliferation and remodeling, all regulated by growth factors, including PDGF-BB, bFGF, EGF, IL-1 and TGF, which stimulate hyaluronan production and modulate its size. Interestingly, TGF induced the synthesis of high molecular mass hyaluronan in dermal fibroblast by suppressing the expression of KIAA1199 (also designated as HYBID, hyaluronan-binding protein involved in hyaluronan depolymerization; CEMIP, cell migration inducing hyaluronan binding protein), but induced hyaluronan of intermediate size in synovial fibroblasts of patients with osteoarthritis or rheumatoid arthritis, because of the inability of TGF to efficiently suppress HYBID [85]. Thus, KIAA1199 may be involved in the catabolism of TGF-synthesized hyaluronan in arthritis synovium and to a lesser extent in the dermis. Furthermore, the TGF-mediated synthesis of hyaluronan in dermal fibroblasts was mediated by up-regulation of *HAS1* and *HAS2* mRNA via Smad3 in a crosstalk with ERK1/2 MAPK [86].

EGF or IL-1 increased the expression of *HAS2/HAS3* genes in cultured oral mucosal cells, but had no effect on *HAS1*. However, these cytokines upregulated *HAS1* and *HAS2* in dermal fibroblast cultures, but not *HAS3* [87] (Table 1). These differences suggest that the regulatory mechanisms and the stability of each of the *HAS* transcripts differ between oral and dermal fibroblasts, which may be of significance to explain why scars rarely form in the mucosa, in contrast to the skin.

The synovial fluid of inflamed joints is characterized by excessive amounts of polydisperse hyaluronan, and the presence of IL1, TNF, and TGF, as well as infiltrating leukocytes TGF was demonstrated to be a potent stimulant of *HAS1* mRNA in human fibroblast-like synoviocyte cultures; this cell type plays an important role in the development of rheumatoid arthritis [88, 89]. TGF potently stimulated *HAS1* transcript induction via activation of p38 MAPK signaling as the main route, and to a less extent via the ERK1/2 MAPK pathway (Table 1). Additional studies both *in vitro* and *in vivo* demonstrated that the accumulation of polydisperse hyaluronan amplify a vicious cycle, i.e. infiltration/activation of leukocytes, release of cytokines, which in turn lead to more accumulation of hyaluronan and activation of leukocytes [90]. Notably, the strong induction of *HAS1* mRNA by IL-1, but not of TGF, was mediated by the NF-kB pathway [91].

Atherosclerosis is linked to accumulation of hyaluronan which promotes proliferation. *HAS1* and *HAS2* were found to be induced by prostaglandins PGE2 and PGI2, in human vascular smooth muscle cells, via Gs-coupled prostaglandin receptors IP and EP2, in a cAMP/PKA-dependent manner. PGE2 is a potent stimulator of hyaluronan production, via a cAMP-dependent signaling in other cell types including fibroblasts [56].

Thus, the amounts and sizes of synthesized hyaluronan differ in response to local regulatory stimuli.

**2.3 Hyaluronan production is connected with** **metabolic diseases**

Biosynthesis of the hyaluronan precursors, UDP-GlcUA and UDP-GlcNAc, requires glucose and is thus affected by the metabolic state of the cell; UDP-GlcUA and UDP-GlcNAc are synthesized as side reactions of the glycolytic pathway starting from glucose-1-phosphate (Glc-1P) and fructose-6-phosphate (F-6P), respectively, both derived from glucose-6-phosphate (Glc-6P) (Fig. 2). Hyaluronan synthesis is regulated by the levels of its precursors [54], as well as the levels of HAS isoforms, which are regulated by growth factors and cytokines (Table 1). Interestingly, HAS2 expression and stability/activity [92], as well as *HAS2-AS1* expression [65], are strongly regulated by *O*-GlcNAcylation which is connected with cellular metabolism and might act as a nutrient/metabolic sensor [93]. Post-translational *O*-GlcNAcylation of cytosolic and nuclear proteins via *O*-linkage of N-acetylglucosamine (*O*-GlcNAc) is linked to oversupply of glucose and obesity. UDP-GlcNAc is the donor sugar for *O*-GlcNAcylation, and is synthesized from glucose, glutamine and UTP, via the hexosamine biosynthetic pathway (HBP) [93, 94] (Fig. 2). *O*-GlcNAcylation, similar to phosphorylation, dynamically modify serine and threonine residues and is added and removed from proteins in response to cellular mediators [95]. The HBP integrates several nutrient inputs in the synthesis of UDP-GlcNAc, however, the molecular mechanisms underlying the flux into the HBP are not well understood. In diabetes, which is associated with increased glucose levels, and in cancer cells with altered metabolism, excess of glucose, free fatty acids, the amino acid glutamine and nucleotide uridine can be funneled into the HBP resulting in the production of UDP-GlcNAc and subsequent *O*-GlcNAcylation. Importantly, the hypoxia-induced factor 1 (HIF-1) which is upregulated in the hypoxic microenvironment of solid tumors, is correlated to hyaluronan overproduction in breast cancer cells via HBP-coupled HIF-1 signaling [96].

**2.4 Coordinated regulation of hyaluronan metabolism at transcriptional and translational levels**

As described above HAS2-mediated synthesis of hyaluronan is regulated at the transcriptional level, but also post-translationally by *O*-GlcNAcylation [54, 65, 97]. The induction of *HAS2-AS1* in response to high glycosamine or glucose, favors *HAS2-AS1*/*HAS2* (RNA/mRNA) heteroduplex promoting HAS2 expression and hyaluronan production [64, 65] (Fig. 3).

Another post-translational modification of proteins is ubiquitination, which affects protein activity, stability and interaction(s) with other protein(s) [98-100]. Recently, we found that HAS2 activity and stability are modulated by ubiquitination [80]. These results demonstrated that the functional form of HAS2 is a dimer and ubiquitination of HAS2 at lysine 190, which is located in the glycosyltransferase domain and is conserved among all HAS isoforms, has a key role for its activity [80, 81]. An interconnection between *O*-GlcNAcylation and ubiquitination has been observed, where the former may regulate the latter; the ubiquitinating enzyme E1, which initiates ubiquitination of proteins, is modified by O-GlcNAc [101, 102]. Protein ubiquitination is reversed by members of a family of approximately 90 de-ubiquitinases (DUBs). Using a DUB cDNA overexpression screen, we identified that USP17 preferentially de-ubiquitinated HAS2 poly-ubiquitination, and that USP4 preferentially de-ubiquitinated HAS2 mono-ubiquitination [103]. Notably, HAS2 stability increased upon *O*-GlcNAcylation of serine 221 (t1/2 of about 5 h) [92], and by de-ubiquitination of lysine 190 (t1/2 of about 36 h) [103]. A possibility that remains to be elucidated, is that if *O*-GlcNAcylation of HAS2 recruits the HAS2 deubiquitinases USP4 and USP17 (Fig. 3).

Tammis´ group found that the binding capacities of the transcription factors SP1 and YY1 to the *HAS2* promoter are regulated by *O*-GlcNAcylation; insulin- and glycosamine-induced *O*-GlcNAcylation of SP1 reduced, whereas *O*-GlcNAc modified YY1 increased the binding to the *HAS2* promoter, activating or suppressing *HAS2* transcription, respectively [75] (Fig. 3). This metabolic control of HAS2 is very important given the increased hyaluronan production in diabetes which among other pathophysiological processes is involved in the development of heart disease and atherosclerotic lessions [94, 104]. Whereas the cellular UDP-GlcNAc fluctuations appear to fine-tune hyaluronan synthesis [75], several questions still remain to be answered, regarding the mechanisms of gene regulation in response to O-GlcNAcylation.

Our earlier studies demonstrated that the stimulatory effects of PDGF and TGF on hyaluronan synthesis occur at the transcriptional level, since the inhibitor of protein synthesis cycloheximide, suppressed the stimulatory effects. However, the powerful hyaluronan stimulatory effect of PMA, an activator of PKC, was virtually unaffected by cycloheximide, suggesting that PKC can directly phosphorylate and activate HAS isoforms, or phosphorylate a component which in turn activates the enzyme(s) [105]. Vigetti and colleagues [106], demonstrated that the energy stress marker AMP-activated protein kinase (AMPK) phosphorylates HAS2 at threonine 110 and inhibits its activity. As hyaluronan synthesis is an energy demanding process, it is possible that AMPK activation under catabolic situations inactivates HAS2, suppressing hyaluronan synthesis. Long-term forskolin treatment, which also activates AMPK [107], resulted in suppression of hyaluronan synthesis in fibroblast cultures, further supporting an inhibitory effect of AMPK on hyaluronan production [105]. These findings illustrate the multifaceted regulation of HAS2 protein stability and activity.

Another level of regulation of the expression of *HAS* genes occurs post-transcriptionally by microRNAs (miRNAs), which upon binding to the 3´UTR of target mRNAs alter gene expression by RNA degradation or translational repression [108]. For example, miRNA23 and Let-7A suppress HAS2 expression (Fig. 3), however, in the interest of space their regulatory control will not discussed further.

**3. Targeted disruption of HAS isoformsand consequences of hyaluronan deficiency**

During early mouse development, *Has1* and *Has2* expression patterns overlap. During mid-gestation, *Has1* expression ceases but *Has2* expression strongly increases to embryonic day 9.5 and is observed in neural crest cells and endocardial cushions. At E10.5, *Has3* expression is restricted to the developing teeth, hair follicles and whiskers; the expression pattern of *Has2* complements that of *Has3*. Thus, *Has2* is the major hyaluronan synthesizing enzyme during embryogenesis and the key source for hyaluronan during mid-gestation. *Has2*-/- mice die at E10.5 due to a cardiovascular defect because Has2-synthesized hyaluronan is needed for the Ras-dependent induction of endocardial to mesenchymal transition, required in valve and septa formation in the heart [4, 109-111]. Interestingly, a mutationof *HAS2* leading to the replacement of glutamic acid 499 to a valine residue results in decreased hyaluronan synthesis, and has been seen in children with ventricular septal defect [112]. Because *Has2*null mice are embryonically lethal, its role has been investigated by conditional inactivation of the gene. In conditional *Has2*-deficient mice, limbs were shortened, revealing a crucial role of hyaluronan in skeletal growth and patterning, maturation of chondrocytes and synovial joint formation [113]. No apparent morphological defect in the brain of mice with conditionally deleted *Has2* was observed [114].

Hyaluronan is an important component of the stem cell niche and a large body of studies have revealed its role in many facets of stem cell biology, including limbal stem cells that have a vital role in maintaining the cornea [115, 116]. Interestingly, compensatory mechanisms were evoked to maintain the level of hyaluronan in the stem cell niche and thus the limbal stem cell phenotype; in mice with conditional *Has2*-deficient corneal epithelium *Has1* and/or *Has3* were upregulated, whereas in *Has1* deficient (*Has1*-/-) and *Has3* deficient (*Has3 -/-*) mice *Has2* expression was upregulated after injury [116]. Thus, hyaluronan microenvironment maintains limbal stem cell phenotype.

Initially, no obvious abnormalities were observed in *Has1*-/- and *Has3 -/-* mice [117]. However, subsequent studies revealed that in double-knockout mice (*Has1/3* null), dermal wound closure was accelerated compared to WT mice, due to creation of a proinflammatory milieu favoring recruitment of neutrophils [118]. Notably, *Has2* expression was increased in these mice, which might have contributed to the increased wound closure. Further studies on the repair process after articular cartilage injury, revealed that *Has1*-/- mice exhibited a chronic joint inflammation, intra-articular scarring and severe osteoarthritis-like symptoms; the impaired repair can be partly explained by enhanced expression of NF-B signaling, fibrosis and apoptosis inducers. The ablation of *Has1* resulted in the induction of tissue metalloproteinase inhibitor 1 and 2 (Timp1andTimp2) which are active during the anabolic phase of osteoarthritis contributing to fibrotic matrix deposition [119]. Importantly, our studies demonstrated that HAS2-mediated breast cancer invasion is promoted by suppression of TIMP1 [120]. Thus, Has1 and HAS2 affect ECM remodeling during chronic inflammation and cancer, respectively, partly in conjuction with Timps/TIMPsexpression.

Although hyaluronan is considered to be the most abundant glycosaminoglycan in adult brain, its neurophysiological functions are not well known. Electrophysiological analyses of *Has* knock-out mice, *Has1*-/-, *Has3 -/-* and brain-targeted conditionally knock-out *Has2* mice, revealed that all *Has* mutant mice exhibited a susceptibility to epileptic seizures. However, the seizure activity was more prevalent in *Has3 -/-* mice along with a decrease in hyaluronan and ECM in the hippocampus, not detected in the other mutant mice. This difference is a key mechanism for epileptic seizures because the diffusion of molecules is affected and reflects a physiological role for hyaluronan in regulating brain ECM [114]. Furthermore, hyaluronan synthesized by Has3 partly accounts for the ventilator-induced lung [121] and intestinal [35] inflammation, as well as neointimal hyperplasia [122], since the observed inflammatory responses were reduced in *Has3 -/-* mice.

**4. Clinical impact of aberrant *HAS* genes expression and excessive hyaluronan production**

**4.1 *HAS1* splice variants can be used as markers for systemic B-cell cancers**

Alternative splicing of genes increases the number of functional protein products, contributing to fine-tuning of gene function [123]. Repeats with the potential to affect *HAS1* splicing were identified [68]. However, very little is known about the tissue expression and role in hyaluronan synthesis of the different splice forms. This is because of the low transcript expression and short lifetime in normal tissues. Expression of the full length *HAS1* (*HAS1-FL*; normal alternative splicing pattern) in CHO cells exhibited a 4-fold and 10-fold lower hyaluronan synthesizing capacity than HAS2 and HAS3, respectively, reflecting differences in the polymerization rate, interaction(s) with different cytoplasmic proteins and/or transcript stability. Furthermore, HAS1 is the least active of the synthases under non-stressed conditions [124]. During mouse embryonic development, the *Has1* gene is temporally expressed during gastrulation and early neurulation [4]; in adult tissues, it is highly expressed in ovary, but also in spleen, thymus, prostate, intestine and testes [60].

Pioneering studies by Adamia S., Pilarski L. and colleagues have identified and characterized three aberrant splice variants of *HAS1* (*HAS1-Va*, *HAS1-Vb*, *HAS1-Vc*) that contribute to oncogenesis and progression of plasma cell dyspasia, in particular multiple myeloma (MM) and Waldenstom macroglobulinemia (WM) [125-129] (Fig. 4). Patients with MM, formed by malignant plasma cells (PCs), have a median survival time of about four years. Given that MM is derived from B cells in the bone marrow, the blood, or both, it is interesting that HAS1 variants expressed by the B cell compartment correlates with poor outcome of MM patients [125, 126]. *HAS1* full lengthandHAS1 variants are absent from healthy donors. They are also largely absent from MM patients non-B / T cell compartment, but highly expressed in the B cell lineage of MM patients. Notably, HAS2 is expressed only by the MM non-B/T cells compartment, whereas HAS3 is expressed by both healthy donors and MM patients (Fig. 4). Interestingly, *de novo* synthesized hyaluronan by HAS1 and HAS3 had a membrane localization, whereas that of HAS1 variants was cytoplasmic [130]. An explanation for oncogenic properties of HAS1 variants is based on the “Goldilocks” hypothesis, i.e. the proteins involved in cell-cycle progression have to be “just right” [125, 131]. To this, contribute also the hyaluronan receptor RHAMM, whose expression and isoform balance correlates to poor outcome in MM [132-134]. It was proposed that RHAMM binding to intracellular hyaluronan synthesized by HAS1-Vs disrupts RHAMM-microtubule interactions permitting the progression through the cell cycle and survival of MM cells.

Other studies have demonstrated that increased expression of HAS1, possibly involving the expression of HAS1-Vs, correlates to poor patient survival in serous ovarian cancer [135], in bladder cancer [136], colon cancer [137] and breast cancer [138]. A recent study revealed that estradiol E2, which has been associated with the progression of cardiovascular diseases in women after menopause, was shown to inhibit HAS1 expression through an ER-dependent inhibition [139] (Table 1). However, it is not only the overexpression, but also the reduced expression, of HAS1 and HAS2, which is associated with poor prognosis in melanomas [140].

**4.2 Biological significance of HAS2-synthesized hyaluronan in breast cancer**

Breast cancer is the most commonly diagnosed malignancy in women and about 30% of patients suffer from metastases in bones, lungs, liver and brain [141]. Ample evidence support a key regulatory role of HAS2-synthesized hyaluronan in breast cancer progression [6, 142]. HAS2 regulates the growth, stemness, invasiveness and metastatic behavior of breast cancer cells [25, 103, 120, 143-145], as well as the resistance of dermal fibroblasts to environmental stress-induced apoptosis [146]. In the triple-negative breast cancer cell line Hs578T which displays a mesenchymal stem-like subtype [147], the ablation of *HAS2* led to a compensatory induction of *HAS1* and *HAS3* expression, synthesis of low molecular mass hyaluronan, reduced cellular growth and invasion [143, 148] . Thus, invasive breast cancer cells upon depletion of *HAS2* evoke mechanisms to maintain their hyaluronan production. Importantly, increased serum low molecular mass hyaluronan in breast cancer patients is correlated to metastasis [149]. Furthermore, knockdown of *HAS2* significantly suppressed the EGF-mediated focal adhesion kinase/PI3K/Akt signaling and thus breast cancer cell invasion [120].

During the metastatic process, epithelial cancer cells adapt an embryonic program to be released from the primary tumor by acquiring a migratory mesenchymal phenotype (epithelial- mesenchymal transition; EMT), and at sites of colonization the reverse process occurs (mesenchymal-epithelial transition; MET) [150]. We have demonstrated that intrinsic HAS2 expression by the metastatic breast cancer clone MDA-MB-231-BM, selected for its capability to metastasize in bone, is required for the EGF-mediated activation of FAK/PI3K/Akt signaling and is reversely correlated to TIMP-1 expression and activity, which promotes invasion [120]. Interestingly, peritumoral hyaluronan-dependent matrices are important for the adhesion of metastatic breast cancer cells to CD44 expressed by microvascular endothelial cells [25]. Thus, HAS2-mediated hyaluronan synthesis by breast cancer cells promotes both their invasive and metastatic phenotype.

Molecularly, EMT is induced by the activation of specific EMT transcription factors, including Twist, Snail1/2 and ZEB1/2; multiple extracellular stimuli including TGF, hepatocyte growth factor (HGF) and epidermal growth factor (EGF) cooperate to drive signal transduction pathways and induction of EMT transcription factors [151, 152]. Earlier studies demonstrated that HAS2-synthesized hyaluronan plays a central role in the transition of epithelium to mesenchyme during embryonic development and acquisition of the malignant phenotype of carcinoma cells. For example, elevated HAS2-synthesized hyaluronan increased the malignant properties of mesothelioma cells, as determined by increased locomotion, induction of proliferation markers cyclin A and B, and anchorage-independent growth, compared to that of non-hyaluronan synthesizing mesotheliomas [153]. Furthermore, HAS2-mediated hyaluronan increase in the human mammary epithelial cells MCF10A, induced a mesenchymal phenotype by stimulating PI3K/Akt and hyaluronan-CD44 signaling [154]. In normal NMuMG mouse mammary epithelial cells, intrinsic HAS2 transcriptional activity was required for full effect of TGF-induced EMT, involving Smad and non-Smad signaling pathways, in a CD44-independent manner. Notably, HAS2 silencing affected the TGF-mediated cell migratory capacity, but not that of EGF, indicating a selectivity of the role of HAS2 in TGF-mediated migration [155]. However, a recent study demonstrated a role of hyaluronan-CD44 signaling in EMT in breast cancer cells expressing high levels of CD44 with high hyaluronan binding capacity; in a self-enforcing feedback loop, HAS2-synthesized hyaluronan activated CD44 to trigger ZEB1 expression which bound to the promoter of *HAS2* further elevating hyaluronan production by activation of *HAS2* [156]. This knowledge, and that in breast cancer cells undergoing EMT there is a cancer stem cell population that is promoted by elevated hyaluronan [145], indicates an important role of HAS2 in breast cancer malignancy.

Studies by us and other laboratories have demonstrated a correlation of hyaluronan production and hormone receptor status of breast cancer cells; hormone negative breast cancers both synthesize and bind hyaluronan [6, 7, 14, 96, 142, 144, 157]. In particular, a correlation has been demonstrated between HAS2 expression and estrogen receptor negative invasive breast cancer subtypes; an about 30% of invasive ductal carcinomas and 73% of metaplastic carcinomas express HAS2 [6, 158, 159]. Interestingly, treatment of MDA-MB-231 cells with 4-methylumbelliferone (4-MU), an established inhibitor of hyaluronan synthesis, significantly reduced hyaluronan accumulation in ECM as well as intracellularly, through down-regulation of HAS2 and the up-regulation of hyaluronidases (HYAL1, HYAL2); the observed changes in hyaluronan content were accompanied by modulation of ECM effectors such as proteases and inflammatory mediators, resulting in a significant attenuation of the metastatic potential of these cells (Karalis et al., in preparation).

Increased levels of hyaluronan have been demonstrated in epithelial and connective tissue of various cancers in the tumor parenchyma and/or tumor stroma, and hyaluronan-stimulatory and growth-promoting factors released by cancer cells modulate hyaluronan synthesis in neighboring mesenchymal cells [5, 160-166]. However, a recent study on breast carcinomas demonstrated a strong correlation of HAS2 expression with cancer cells, but only a minor expression in stromal cells [159].

In humans, the *HAS2* gene exhibits a predisposition to undergo chromosomal rearrangements and fuse with other genes. Such gene rearrangements might be early molecular events during the transformation of epithelial cells. The *PLAG1* oncogene, which is the cause for transformation of adipocytes and is associated with lipoblastomas, is fused to the *HAS2* 5´untranslated region; *HAS2-PLAG1* is under the control of *HAS2* promoter and is most likely transcriptionally activated by *IGF2* [167]. Notably, lipoblastomas are characterized by stromal myxoid changes containing hyaluronan, and notably such hyaluronan-rich myxoid changes in breast carcinomas are associated with tumor invasion and metastasis [168]. Interestingly, in radiation-transformed breast cancer cells the *HAS2* gene exhibited a prevalence for chromosomal rearrangements and deregulated expression; the *HAS2* gene was translocated to the *glutamate receptor GRID1*-containing region on chromosome 10, but the DNA sequence which is fused to could not be identified [169]. *HAS2* rearrangements occurred in about 28% of sporadic breast cancer and might reflect the importance of HAS2 activity in early stages of breast cancer, and offers a potential for therapeutic intervention [170-172]. Notably, the demonstrated strong expression of HAS2 in the myoepithelial border of columnar cell lesions, but not terminal duct-lobular units, suggests an important role of myoepithelial-derived HAS2 in the pathogenesis of columnar cell lesions [159]. Furthermore, post-translational O-GlcNAcylation of HAS2 and HAS3, which stabilizes the proteins and correlates with increased stromal hyaluronan, increased relapse and distant metastasis of breast cancer [172, 173]. Thus, HAS2-synthesized hyaluronan promotes breast cancer aggressiveness.

**4.3 Hyaluronan as a marker and therapeutic target in virus infection**

Not only growth factor and cytokine activities are linked to hyaluronan synthesis, but viruses infection-mediated inflammation is also perpetuated through reinforced hyaluronan-CD44 interactions between mesenchymal cells and immune activated mononuclear leukocytes [174]. De la Motte and colleagues demonstrated that the respiratory syncytial virus infection of smooth muscle cells was correlated to mononuclear cell adhesion and accumulation of hyaluronan [175] (Table 2). More recently, Stuhlmeier demonstrated that excessive accumulation of hyaluronan in synoviocytes is a consequence of Epstein-Barr virus infection through the induction of *HAS1* mRNA via NF-B pathway, but also other double and single-stranded viral RNA analogs [89]. Furthermore, increased serum hyaluronan levels and disease severity has been demonstrated after infection by, for example, Kaposi sarcoma-associated herpesvirus [176] and hepatitis C virus [177]. Human immunodeficiency virus (HIV-1) upregulate the transcriptional activity of *HAS1* [178]. On the other hand, knockdown of HAS2 enhanced HIV-1 [179] (Table 2). Not only viral infections, but also bacterial infections lead to increased serum hyaluronan levels [180].

Recently, increased serum hyaluronan levels have been associated with dengue virus infection and disease severity [181]; however, a relatively small number of patients were investigated at their recovery phase. More recently, we followed up a large cohort of patients from the onset of infection to the recovery phase. The analysis defined serum hyaluronan levels as a marker for the development of dengue warning signs and severe dengue (Lin, et al., manuscript in preparation). Given that dengue is a common and rapidly spreading virus because of increased traveling and global warming, the elucidation of molecular mechanisms underlying the aberrant accumulation of hyaluronan and its functional significance are important to investigate. An important question to elucidate is if the newly synthesized hyaluronan initially has a protective role or not, acting as a proinflammatory mediator during the progress of the infection.

1. **Conclusions and Perspectives**

There is ample evidence that hyaluronan synthesized by the three HAS isoforms differ functionally depending on the context and the stimuli involved. Because elevated hyaluronan amounts are essentially associated with all disease processes involving tissue damage, including persistent inflammation and cancer progression, better understanding of the mechanisms of activation and regulation of each one of the three HAS isoforms is imperative for a better understanding of hyaluronan in pathological conditions. To this end, an analysis of the genomic regulation of the *HAS* genes in response to external or cellular signals is required. Furthermore, the role of post-translational modifications affecting the stability and activity of HAS isoforms have to be elucidated. In addition, the roles of hyaluronan-mediated signaling, through its receptors in the regulation of cellular fate need to be unravelled. Such knowledge about hyaluronan biology may unravel therapeutic opportunities in inflammation and cancer.

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**Figure legends**

**Fig. 1. Transcription factor binding sites in the promoters of *HAS* and *HAS2-*AS1 genes.** Response elements were identified *in silico* and/or experimentally. Numbers are relative to the transcription start site (TSS). Relevant references are indicated.

**Fig. 2. Biosynthetic pathway of hyaluronan and *O*-GlcNAcylation**

The precursors of hyaluronan, UDP-GlcUA and UDP-GlcNAc, are synthesized as side reactions of the glycolytic pathway. UDP-GlcNAc can be used both in the synthesis of hyaluronan and can be transferred to Ser or Thr residues of proteins resulting in *O*-linked GlcNAc (*O*-GlcNAcylation). 4-MU binds to glucuronic acid instead of UDP, inhibiting the synthesis of hyaluronan by HAS isoforms. HAS, hyaluronan synthase; 4-MU, 4-methylumbelliferone; Glc, glucose; UDP, uridine diphosphate; GlcUA, glucuronic acid; P, phosphate; F, fructose; HBP, hexosamine biosynthetic pathway; GlcN, glucosamine; GlcNAc, N-acetylglucosamine; OGT, O-linked glycosylation transferase.

**Fig. 3. Control of HAS2 activity** **at transcriptional and translational levels**

External stimuli through specific cell surface receptors induce the transcriptional activity/ inhibition of *HAS2-AS1* and/or *HAS2* by *O*-GlcNAcylation (G). *HAS2* transcriptional expression is also regulated by the IL1/PDGF-responsive *SMILR*. Posttranscriptional regulation of *HAS2* mRNA by miR23 and Let-7 also occurs. *O*-GlcNAcylation of HAS2, at serine 221 (S221), stabilizes the HAS2 protein. HAS2 phosphorylation at threonine 110 (T110) by AMPK inhibits its activity while phosphorylation through PKC leads to its activation. Ubiquitination of HAS2 at lysine 190 (K190) is important for its activity and the de-ubiquitinase USP17 removes polyubiquitin chains leading to HAS2 stabilization, whereas USP4 removes monoubiquitination inhibiting its activity.

**Fig. 4. Aberrant splicing of *HAS1* and expression profiles of *HAS* genes in MM patients.**

Alternative splicing, within exons 3,4 and 5, of full length *HAS1* results in *HAS1* variants; *HAS1Va*, skips exon 4; *HAS1Vb,* skips exon 4 and retains 59 base pairs from intron 4; *HAS1Vc,* retains 26 base pairs from intron 4. All splice variants are truncated proteins possessing premature termination codons (PTC; TAA). TGA, termination codon.

++, high expression; -/+, low expression; -, not expressed.